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Molecular Cloning of Abscisic Acid-Responsive mRNAs Expressed during the Induction of Freezing Tolerance in Bromegrass (*Bromus inermis* Leyss) Suspension Culture[1]

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Abscisic acid (ABA) increases the freezing tolerance of bromegrass (*Bromus inermis* Leyss) cell-suspension cultures at 23°C and elicits many metabolic changes similar to those observed during cold acclimation. Induction and maintenance of freezing tolerance by ABA is accompanied by the expression of novel polypeptides and translatable RNAs. The objective of this study was to isolate and characterize ABA-responsive cDNAs associated with ABA-induced freezing tolerance in bromegrass cell cultures. Among the 16 ABA-responsive cDNA clones isolated, 9 were expressed only with ABA treatment, 7 showed increased transcript level, and 1 was transiently expressed. Cold responsiveness was determined in three clones with increased transcript levels and in the transiently expressed clone. Deacclimation of ABA-hardened cells was a relatively slow process, because all of the novel transcripts persisted for at least 7 d after cells were cultured in ABA-free medium. Preliminary sequencing of cDNAs has identified several clones that share high sequence homology with genes associated with sugar metabolism, osmotic stress, and protease activity. Clone pBGA61 was fully sequenced and tentatively identified as an NADPH-dependent aldose reductase. The predicted amino acid sequence of the coding region shared 92% similarity with that predicted for barley aldose reductase cDNA. It is proposed that expression of genes related to sugar metabolism and osmotic stress may be required for ABA-induced hardening.

Many temperate plant species develop greater freezing tolerance when exposed to low, nonfreezing temperatures. This process, cold acclimation, involves a variety of biochemical and physiological changes resulting in the induction of freezing tolerance (Levitt, 1980). Accumulating evidence indicates that changes in gene expression occur during cold acclimation (Guy, 1990; Thomashow, 1990). cDNAs corresponding to novel RNAs expressed during cold acclimation have been isolated from a number of plant species (Mohapatra et al., 1988, 1989; Cattivelli and Bartels, 1990; Hajela et al., 1990; Kurkela and Franck, 1990; Nordin et al., 1991; Houde et al., 1992; Luo et al., 1992).

In addition to low temperatures, exogenous application of ABA to either whole plants (Chen et al., 1983; Lang et al., 1989) or cell cultures (Chen and Gusta, 1983; Keith and McKersie, 1986) at room temperature also induces freezing tolerance. With bromegrass (*Bromus inermis*) cell cultures, it has been demonstrated that 4 to 5 d of ABA treatment increases freezing tolerance by 23°C, with a change in LT_{50} from −7 to −30°C (Chen and Gusta, 1983; Lee et al., 1991). Associated with a rapid increase in hardiness were qualitative changes in the population of polypeptides (Robertson et al., 1987) and translatable RNAs (Lee et al., 1991). Deacclimation of ABA-hardened bromegrass cells is a relatively slow process (Reaney et al., 1989; Lee et al., 1991) compared to other cold-hardy species (Guy et al., 1985; Hajela et al., 1990; Tseng and Li, 1990). Correspondingly, the disappearance of novel polypeptides and RNAs was slow (Lee et al., 1991). It is not clear in this system whether freezing tolerance induced by ABA or by low temperature occurs via the same mechanism.

The objective of this study was to isolate and characterize ABA-responsive cDNAs associated with ABA-induced freezing tolerance. Differential screening of a cDNA library constructed from poly(A)$^+$ RNA of ABA-treated bromegrass cells resulted in the identification of 16 ABA-responsive cDNA clones. Using the EST method of Adams et al. (1991), we have identified several cDNA clones with high nucleotide sequence homology with genes involved with sugar metabolism, desiccation tolerance, and protease activity. Clone pBGA61 was completely sequenced and was found to share a high degree of similarity to the barley NADPH-dependent aldose reductase cDNA (Bartels et al., 1991).

MATERIALS AND METHODS

Plant Material and Treatments

Cell-suspension cultures of smooth bromegrass (*Bromus inermis* Leyss) were maintained and subcultured as described by Chen and Gusta (1983). Five-day-old cells were treated with various concentrations of (±)-ABA in medium containing 0.5 mg L^{-1} of 2,4-D and incubated at 23°C on an orbital shaker for various times. Deacclimation after ABA treatment was initiated by thoroughly washing cells with ABA-free medium and transferring them into fresh ABA-free medium (Lee et al., 1991). Cells were cold treated by incubating at 4°C for up to 5 d while shaking. A sample of the cells from

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Abbreviations: EST, expressed sequence tag; LT_{50}, temperature at which 50% of the cells were killed; SSC, standard sodium citrate.

each treatment was collected to determine the level of freezing tolerance (Lee et al., 1991). The remaining portion was immediately frozen in liquid nitrogen and stored at −70°C. All treatments and freezing tests were repeated three times.

RNA Isolation

As detailed by Lee et al. (1991), total RNA was extracted by the guanidine isothiocynate/cesium chloride ultracentrifugation method. Poly(A)$^+$ RNA was further separated by the push column method (Lee et al., 1992).

cDNA Library Construction and Differential Screening

Poly(A)$^+$ RNA (5 μg) from bromegrass cells treated with 75 μM ABA for 5 d was used for the construction of a cDNA library. Double-stranded cDNA was synthesized by the linker-primer/RNase H method of Gubler and Hoffman (1983) and cloned into the *EcoRI/XhoI* site of the λZapII vector (Stratagene, La Jolla, CA). Primary recombinant clones were packaged in vitro with Gigapack II (Stratagene) and subjected to one round of amplification in *Escherichia coli* strain PLK-F′.

Recombinant phage were plated at a density of 5 × 10^4 plaque-forming units/132-mm plate with *E. coli* strain XL1-Blue (Stratagene). Duplicate plaque lifts were made on nitrocellulose filters (Hybond-C; Amersham, Arlington Heights, IL). First-strand [^{32}P]dCTP-labeled cDNA probes were made from poly(A)$^+$ RNA (Sambrook et al., 1989) of control or ABA-treated cells and then hybridized to plaque lifts. Prehybridization and hybridization conditions were 6× SSC (1× SSC = 0.15 M NaCl, 0.015 M Na citrate), 0.06× Blotto (1× = 5% [w/v] nonfat dried milk, 0.02% NaN_3), and 10 μg μL^{-1} of poly(A)$^+$ RNA at 65°C. Membranes were washed three times in 2× SSC containing 0.1% SDS at 65°C for 20 min and once in 1× SSC, 0.1% SDS for 30 min and then exposed to x-ray film (Kodak XAR-5) with one intensifying screen for 36 h.

Plaques that differentially hybridized to the probe from ABA-treated cells were selected and purified. Positive clones were cross-hybridized to select unique clones. The phagemid containing the cDNA was rescued by the in vivo excision method according to the manufacturer's directions (Stratagene).

Analysis of RNA

Total RNA (20 μg) from ABA- or cold-treated bromegrass cells was slot blotted (Sambrook et al., 1989) onto nitrocellulose membranes (Bio-Rad), prehybridized, hybridized, and washed using the same conditions described for plaque lifts. Each of the cDNA probes were [^{32}P]dCTP labeled by the random priming method and hybridized individually to the blotted membrane. Autoradiographs of membranes were scanned with a densitometer (model 1650; Bio-Rad) to determine relative steady-state transcript levels.

Total RNA from treated bromegrass cells was electrophoresed on a 1.3% agarose-formaldehyde gel (Sambrook et al., 1989). After electrophoresis, RNA was stained with ethidium bromide and visualized with UV light to ensure equal loading in each lane. RNA was blotted onto a nylon membrane (Zetaprobe; Bio-Rad) according to the manufacturer's directions. Replicate blots were probed with each unique cDNA isolated. Prehybridization and hybridization were carried out in 0.25 M Na_2HPO_4 (pH 7.2), 1 mM EDTA, 7% SDS at 65°C. Washes were conducted in 5% SDS, 20 mM Na_2HPO_4, 1 mM EDTA at 65° for 30 min followed by two washes in 1% SDS, 20 mM Na_2HPO_4, 1 mM EDTA at 65°C. Membranes were then exposed to x-ray film with one intensifying screen for 2 to 24 h. A randomly selected clone from the bromegrass cDNA library identified a gene expressed at high levels in controls and minimally affected by ABA or cold treatment. As a probe, this clone was used to assure that approximately equal concentrations of RNA were loaded for each gel blot analysis.

Generation of ESTs and DNA Sequencing

Phagemids containing bromegrass cDNAs were purified from overnight cultures by the plasmid column purification method (Stratagene). Approximately 1.5 μg of phagemid in 6 μL of H_2O was used for automated dideoxynucleotide chain termination (Sanger et al., 1977) sequencing with the Applied Biosystems model 373A DNA sequencer. The sequencing strategy was based on the method of Adams et al. (1991). Between 350 and 400 bases of the 5′ end were sequenced, providing enough information for preliminary identification of similar sequences stored in GenBank and EMBL data bases.

A complete nucleotide sequence was obtained for one bromegrass cDNA by the nested deletion procedure of Heinikoff (1987). Each deletion construct was sequenced as described above. The complete cDNA sequence was used to search GenBank and EMBL data bases for similarity matches.

RESULTS

Freezing Tolerance

The frost hardiness of bromegrass cells after various treatments, expressed as LT_{50} values, are summarized in Table I. ABA treatment (75 μM) for 1, 2, or 5 d increased freezing tolerance from −6.5 to −9.4, −12.2, or −30.8°C, respectively. After 5 d of ABA treatment, removal of ABA from the medium did not result in a noticeable decrease in freezing tolerance immediately. However, 7 d after ABA removal, the LT_{50} decreased from −30.8 to −17.1°C. There was no increase in hardiness after 1 d of 0.1 (−6.5°C) or 1 μM (−6.4°C) ABA treatment, but ABA concentrations >10 μM increased hardiness (−7.1, −9.4, and −8.5°C for 10, 75, and 100 μM ABA, respectively). Cold treatment after 1 d did not increase hardiness, but after 5 d, hardiness increased to −15.3°C (Table I).

Isolation of ABA-Responsive cDNA Clones

Poly(A)$^+$ RNA from bromegrass treated with 75 μM ABA for 5 d was used for the construction of a cDNA library. Approximately 4.5 × 10^5 recombinant phage were differentially screened for ABA-responsive clones using first-strand cDNA probes synthesized from poly(A)$^+$ RNA of ABA-treated or control cells. Cross-hybridization detected 16 unique clones of the 100 cDNA clones isolated.

Table I. *Freezing tolerance of bromegrass culture cells after ABA or low-temperature treatment*

Bromegrass cells were incubated with 0 or 75 μM ABA at 23°C or without ABA at 4°C. LT_{50} values were assessed by the 2,3,5-triphenyltetrazolium chloride reduction assay as described in "Materials and Methods." Values represent the means ± SE of three separate experiments.

Treatment		LT_{50}					
ABA concentration	Temp	Days of treatment					
		0	1	2	4	5	12[a]
μM	°C	°C					
0	23	−6.5 ± 1.1	−6.8 ± 0.8	−7.5 ± 0.6	−9.2 ± 1.2	−7.7 ± 1.3	−6.8 ± 1.5
0	4	—[b]	−7.1 ± 1.6	—	—	−15.3 ± 2.8	—
75	23	—	−9.4 ± 0.9	−12.2 ± 1.2	—	−30.8 ± 2.1	−17.1 ± 3.2

[a] ABA was removed on day 5 and cells were cultured for 7 more d in ABA-free medium. [b] —, No data.

RNA Gel and Slot Blot Analysis

To verify ABA responsiveness of genes corresponding to these cDNAs, RNA slot blot analyses of total RNA from control and ABA-treated cells (75 μM; 1-, 2-, or 5-d treatment) were performed. All 16 cDNA clones were identified as ABA responsive (data not shown).

RNA gel blot analysis of total RNA after 0, 1, 2, or 5 d of ABA treatment and of RNA from deacclimated cells previously ABA treated for 5 d was performed using ABA-responsive cDNAs as probes. Three general patterns of ABA-induced gene expression were identified by this method and are illustrated in Figure 1. In the first pattern (pattern A; Fig. 1A), RNA of the corresponding clones accumulated to high levels after 1 d of ABA treatment and continued to increase with 2- or 5-d treatments. Immediately after residual ABA was washed from cells and the cells were cultured in ABA-free medium, the transcript level decreased (Fig. 1, 0d ABA Deacc.) with no noticeable decrease in hardiness (data not shown). Seven days of deacclimation (Fig. 1, 7d ABA Deacc.) reduced both the level of hardiness and the intensity of the hybridization signal. None of the transcripts in this group appeared to be cold responsive after 1 or 5 d of cold treatment. All of the pattern A genes required a minimum of 1 μM ABA to induce accumulation of steady-state RNA.

The second pattern of ABA-responsive gene expression (pattern B) includes transcripts that were expressed in control cells throughout 5 d of treatment but were present at elevated levels during ABA treatment (Fig. 1B). Transcript levels of the 0-d control (Fig. 1B, 0d ck) for pBGA12, pBGA56, pBGA72, and pBGA85 were greater than 1 d after transferring to fresh medium (1d ck). All pattern B gene transcript levels decreased to control levels after 7 d of deacclimation. Three of the transcripts (pBGA12, pBGA56, pBGA85) increased 2- to 3-fold in response to cold treatment (Fig. 1B, 1d +4C and 5d +4C). However, higher steady-state transcript levels were only transient, i.e. there is a higher level of transcript at 1 d than at 5 d. ABA, 0.1 μM, was sufficient to induce a detectable accumulation of transcript, suggesting a high sensitivity of these genes to ABA treatment.

A third pattern of ABA-responsive gene expression (pattern C) identified was similar to pattern B, but increased transcript accumulation was transient (Fig. 1C). Increased transcript corresponding to pBGA25 during ABA treatment was not maintained throughout the treatment period. The transcript levels were elevated after 1 or 2 d but not after 5 d of treatment. Cold treatment increased transcript accumulation after 1 d but not after 5 d.

Similarity of ESTs to Other Genes

ESTs were determined for each bromegrass cDNA, and searches for sequence similarities in both GenBank and EMBL data bases were performed using the Intelligenetics Suite (Intelligenetics, Mountain View, CA). Six bromegrass ESTs with high (>50%) homology to reported sequences were identified (Table II). Of interest are clones pBGA61, pBGA55, pBGA56, pBGA85, and pBGA86 with nucleic acid sequence matches to barley NADPH-dependent aldose reductase (Bartels et al., 1991), barley dehydrin (Close et al., 1989), wheat germin (Lane et al., 1991), human cathepsin D (Faust et al., 1985), and barley embryo globulin (G.R. Heck, unpublished data), respectively, and clone pBGA72 with amino acid sequence matches to rabbit phosphoglucomutase (Ray et al., 1983).

Bromegrass Aldose Reductase Nucleotide and Amino Acid Sequence

To determine whether bromegrass ESTs correspond to predicted homologs of other genes, clone pBGA61 was selected for further analysis. The cDNA insert in pBGA61 was completely sequenced in both directions and determined to be a full-length copy (1192 bp) based on the length of the RNA (1.1 kb) to which it hybridized. The coding strand and predicted amino acid sequence are listed in Figure 2. The longest open reading frame starts at base 79, ends at base 1041, and specifies a polypeptide of 35,938 D, isoelectric point 6.28. Based on percentage of molecular mass, the most abundant amino acids are Lys (11.5%), Glu (9.1%), Leu (8.9%), Val (6.2%), Asp (6.2%), and Ala (4.7%). There does not appear to be a bias for any specific amino acid.

A search made with the complete pBGA61 cDNA sequence strongly suggests its identity as an NADPH-dependent aldose reductase (Bartels et al., 1991). This association is based on 92% similarity found in nucleotide-coding region and predicted amino acid sequence with respect to the putative coding region of the barley cDNA clone pG22-69 (Bartels et

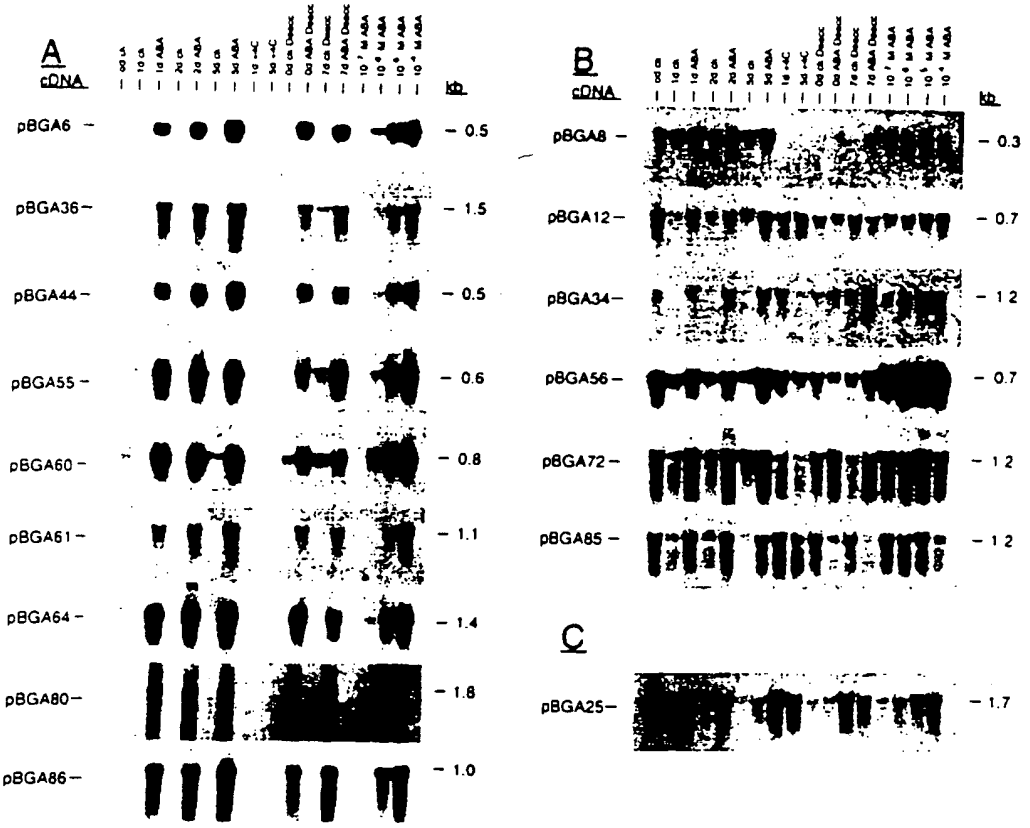

Figure 1. RNA gel blot of steady-state transcript levels after ABA or cold treatment. Bromegrass cells were treated for 0, 1, 2, or 5 d without (0d ck, 1d ck, 2d ck, 5d ck) or with 75 μM ABA (1d ABA, 2d ABA, 5d ABA). Five days after control or ABA treatment, cells were washed with ABA-free medium and immediately frozen for RNA extraction (0d ck Deacc., 0d ABA Deacc.) or cultured in fresh medium for 7 additional d (7d ck Deacc., 7d ABA Deacc.). Cultures were also treated with 0.1, 1.0, 10, or 100 μM ABA for 1 d, and the RNA was extracted for analysis. Twenty micrograms of total RNA was separated on a 1.3% agarose-formaldehyde gel, blotted onto nylon membrane, and probed with a labeled ABA-responsive cDNA. Three ABA-responsive transcript expression patterns are shown: A, Transcript detected only after ABA treatment; B, transcript present in controls but elevated after treatment; C, similar to pattern B but increased transcript level is transient.

ble II. *Bromegrass ESTs with high nucleic acid or amino acid similarity to genes from other organisms*

ESTs of the 16 bromegrass cDNAs were used to search both GenBank and EMBL data bases. Identification refers to the name of the quence matching the EST; Percentage NA and percentage AA indicate the percentage of nucleic acid or amino acid match, respectively;). of bases is the number of contiguous nucleotides used for the sequence search; No. and Ref. refer to the GenBank accession number nless otherwise noted) and corresponding literature citation of the matching sequence, respectively.

EST	Identification	Organism	Percent NA	Percent AA	No. of Bases	No.	Ref.
pBGA55	Dehydrin	Barley	82	55	364	X15287	Close et al., 1989
pBGA56	Germin	Wheat	55	45	408	M63224	Lang et al., 1989
pBGA61	Aldose reductase	Barley	89	89	351	X57526	Bartels et al., 1991
pBGA72	Phosphoglucomutase	Rabbit	—[a]	69	100[b]	A01175[c]	Ray et al., 1983
pBGA85	Cathepsin D	Human	53	53	431	M11233	Faust et al., 1985
pBGA86	Embryo globulin	Barley	76		456	M64372	G.R. Heck, unpublished data

[a] The nucleic acid sequence was not available for this gene. [b] Number of contiguous predicted amino acids used for the rch. [c] Accession no. from the PIR data base.

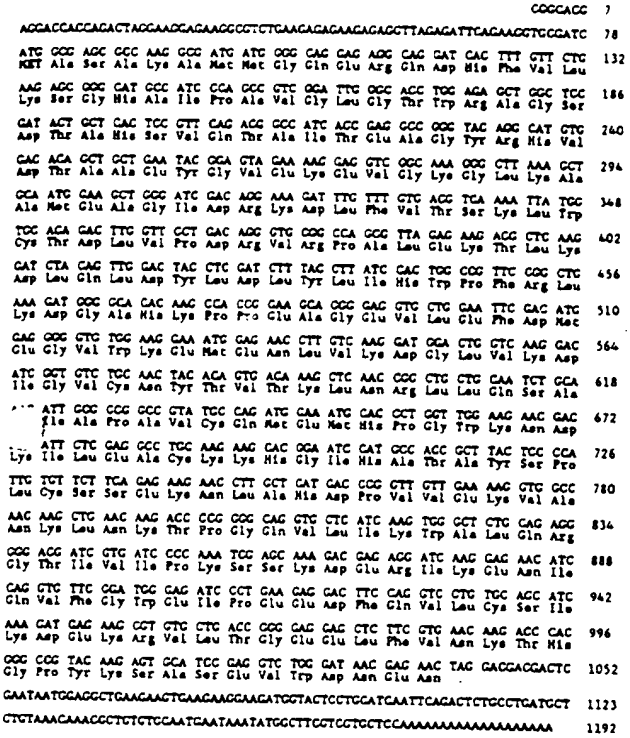

Figure 2. Nucleotide and predicted amino acid sequence of the pBGA61 cDNA insert. The longest open reading frame starts at base pair 79 and ends at 1041.

al., 1991). Nucleotide similarity of the 3'-untranslated region between the two clones was considerably lower (approximately 50%). The putative amino acid sequence of pBGA61 is compared to those of barley pG22-69 and human lens aldose reductase in Figure 3 (Nishimura et al., 1990). The amino acid match between pBGA61 and pG22-69 is high (92%), but neither is as high as with the human lens cDNA.

DISCUSSION

Studies in the past have identified a host of metabolic changes associated with cold acclimation (for review, see Guy, 1990). These changes have been observed at the protein and translatable RNA levels, however, only a small number of these RNAs have been cloned. In this study, we have isolated 16 unique cDNA clones from an ABA-treated bromegrass cDNA library. Transcript accumulation corresponding to these cDNAs can be categorized into three distinct patterns. The first pattern (A) includes transcripts that were detected primarily after ABA treatment. This type of response is similar to that of the cold-responsive genes identified in other species (Robertson et al., 1987; Mohapatra et al., 1988, 1989; Cattivelli and Bartels, 1990; Hajela et al., 1990; Kurkela and Franck, 1990; Luo et al., 1992). With the second pattern (B) of gene expression, transcripts were detected in the controls but were up-regulated by ABA or cold treatment. In the third pattern (C), the increased transcript signal is transient.

Although not all ABA-inducible genes may be involved in the hardening process, expression of some of these genes could be required for development of frost tolerance and be responsible for the numerous physiological and biochemical changes detected during hardening (Levitt, 1980). A decrease in steady-state transcript levels, corresponding to the ABA-induced cDNAs during dehardening, further suggests that some of these genes could be associated with freezing tolerance. Although deacclimation was apparent, as indicated by dehardening and RNA level attenuation, the process was still much slower relative to those in *Arabidopsis* (Hajela et al., 1990; Kurkela and Franck, 1990) at the transcript level and spinach (Guy et al., 1985), alfalfa (Mohapatra et al., 1987; Luo et al., 1992), and potato (Tseng and Li, 1990) at the translatable RNA level. This difference in response time may reflect a difference between monocots and dicots or between cell cultures and whole plants, or it may occur because bromegrass is much hardier than the other species studied. Nevertheless, persistence of specific ABA-induced transcripts may be required for maintaining an elevated level of hardiness.

Previous reports have shown that certain translatable RNAs expressed in bromegrass (Lee et al., 1991) and potato (Tseng and Li, 1990, 1991) during the first few hours of ABA or cold treatment were not present in later treatments. Transient gene expression during hardening may indicate that the gene product represents an intermediary step in hardening. These types of genes, as represented by clone pBGA25, may not directly confer tolerance to freezing but may be components of a cascade pathway ultimately leading to a hardening response.

It was observed that some of the pattern B RNA gel blots revealed a higher transcript level immediately after transfer to fresh ABA-free medium (Fig. 1, 0d ck) than after 1 d of culture in the same medium (Fig. 1, 1d ck). After 1 d of culturing, however, the transcript level was much lower than the level determined after 1 d of ABA treatment. A stronger hybridization signal in the 0-d control is likely due to stress to the cells when they are transferred to fresh medium where the osmotic potential or pH may be different.

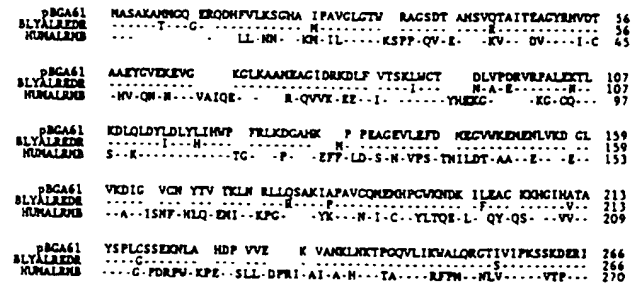

Figure 3. Comparison of the bromegrass cDNA (pBGA61) putative amino acid sequence with that of barley (BLYALREDR, Bartels et al., 1991) and human lens (HUMALRMB, Nishimura et al., 1990) putative aldose reductase sequences. A dash (-) indicates an amino acid match with pBGA61. Gaps were introduced to optimize sequence alignment.

Several of the ABA-responsive bromegrass genes appear o be cold responsive but only after 1 d of cold treatment. ecause the accumulation of transcripts corresponding to nany of these clones was sensitive to subculturing, perhaps recovery period of 1 d at 4°C following transfer would ave resulted in the identification of some false-positive gnals. Therefore, only clones pBGA12, pBGA56, pBGA85, nd pBGA25 are probable representatives of cold-responsive enes. However, the increase in transcript levels correspond- g to these cDNAs was only 2- to 3-fold, which may be tributable to increased RNA stability rather than induced xpression. Additional experiments are needed to confirm the old responsiveness of these genes. Because freezing toler- nce can be induced by either ABA or cold treatment, those BA- and cold-responsive genes may have important roles cold acclimation.

This study demonstrated that exogeneous ABA application ters the expression pattern of many ABA-responsive genes. he function of many of these genes remains unknown. hrough RNA gel blot analysis, it has been possible to emonstrate that many of the ABA-responsive bromegrass enes are associated with development of freezing tolerance. y generating ESTs for the 16 bromegrass cDNAs, we were ble to associate some of them to homologous nucleotide quences of previously sequenced genes. As shown in Table clones share high homology to barley NADPH-dependent dose reductase (Bartels et al., 1991), barley dehydrin (Close al., 1989), wheat germin (Lane et al., 1991), human ca- epsin D (Faust et al., 1985), barley embryo globulin (G.R. eck, unpublished data), and rabbit phosphoglucomutase ay et al., 1983). Both the pBGA61 EST and the putative ding region of the pBGA61 cDNA insert share a high egree of predicted amino acid sequence similarity (89 and %, respectively) to barley aldose reductase. A similar strat- y utilizing ESTs has been used to screen a potato cDNA rary. Three cDNAs, putatively identified as osmotin-like nes, were fully sequenced and found to share a similar gree of homology to tobacco osmotin (B. Zhu, personal mmunication). Therefore, we recommend the EST method dams et al., 1991) as a useful tool for rapidly identifying nes that share homologous sequences to previously re- rted gene sequences.

Aldose reductase (EC 1.1.1.21) catalyzes the stereospecific duction of Glc to sorbitol (Shiono et al., 1987). Bartels et (1991) have cloned an ABA-inducible, aldose reductase- lated protein that is expressed during the desiccation phase embryogenesis in barley. The association of this gene with gar metabolism during desiccation in barley and with ABA- duced freezing tolerance in bromegrass is of significance. st, sugars accumulate during cold acclimation in many ecies (Levitt, 1980), including bromegrass (Tanino et al., 90). Also, cryoprotection studies have demonstrated that rbitol is one of the most effective cryoprotectants (Chen et . 1984). It has been suggested that there is a requirement the up-regulation of genes involved with sugar metabo- m during cold acclimation (Guy, 1990). Evidence for this s demonstrated in winter wheat, where Suc synthase lypeptide and poly(A)$^+$ RNA levels increased with low- nperature treatment (Crespi et al., 1991). Results of this dy also suggest that a phosphoglucomutase gene, encod- ing an enzyme of the glycolysis/gluconeogenesis pathway has a higher level of expression during ABA-induced hard ening. The up-regulation of aldose reductase and phospho glucomutase in bromegrass and of Suc synthase in whea may, therefore, indicate changes in the molecular regulatio of sugar metabolism during hardening.

Expression of a dehydrin-like gene in bromegrass durin ABA-induced freezing tolerance may suggest that hardenin involves changes in tolerance to dehydration stress. Desic cation of barley seedlings induces the expression of dehydri (Close et al., 1989) and like expression of many of the Ral (responsive to ABA) genes expression of dehydrin is alsc induced by ABA treatment (for review, see Skriver anc Mundy, 1990). Rab genes are expressed concurrently witl increased tolerance to water stress; however, the function o these genes is not known. Freezing stress is considered a form of desiccation stress, because extracellular freezing causes cellular water to move outside the cell (Levitt, 1980) It has been demonstrated that ABA-treated bromegrass sus- pension culture cells have greater tolerance to freezing stress and to salt stress (Reaney et al., 1989). It is likely that they also have greater resistance to desiccation stress.

A putative homolog (pBGA56) of the desiccation-related wheat germin gene (Lane et al., 1991) is expressed during ABA-induced hardening in bromegrass. Wheat germin is expressed in partially hydrated (60% water) germinating em- bryos (Lane et al., 1991). The function of this protein is not known; however, a polypeptide from barley that is specifi- cally salt-stress inducible (Hurkman et al., 1991) has a high sequence similarity to it. Germin also has moderate amino acid similarity to spherulins of *Physanum polycephalum*, pro- teins expressed if plasmodia are subjected to environmental stresses, including osmotic and temperature extremes (Lane et al., 1991). Germin-like genes, including the bromegrass gene represented by clone pBGA56, may thus represent members of a class of genes associated with desiccation-stress resistance.

A low-temperature-inducible thiol protease cDNA has been cloned from tomato fruit (Schaffer and Fischer, 1988). The tomato cDNA shares regions of homology with other plant proteases and with the animal protease cathepsin H. The bromegrass pBGA85 clone is not only ABA responsive, but is also cold responsive, like the tomato thiol protease gene. Schaffer and Fischer (1988) have suggested that low temperatures may denature certain polypeptides, thus creat- ing a requirement for proteases to degrade the denatured polypeptides. Cold- and ABA-induced protease expression may, therefore, play a more important role in supporting cellular metabolism during low-temperature growth than in developing hardiness.

Although the identities of aldose reductase, dehydrin, ger- min, and phosphoglucomutase-like RNAs expressed in hard- ening bromegrass remain to be confirmed, the expression of these types of genes may be indicative of a requirement for tolerance to desiccation stress manifested by freezing tem- peratures. Work is in progress to characterize these genes. It is certainly possible that expression of these genes is required for the accumulation of osmolytes and tolerance to extracel- lular freezing-induced dehydration stress.

Clone pBGA86, on the other hand, shares high homology

with barley embryo globulin (G.R. Heck, unpublished data) and with a class of maize embryo proteins (Belanger and Kriz, 1989). Because the maize globulins most likely function as storage proteins (Belanger and Kriz, 1989), clone pBGA86 probably has a similar role and is, therefore, not related to cold acclimation.

In summary, we have isolated 16 cDNAs from bromegrass cDNA library representing genes expressed during ABA-induced freezing tolerance. Three distinct patterns of gene expression have been identified. EST analysis indicates that some of the bromegrass cDNAs have high sequence similarity to barley aldose reductase, rabbit phosphoglucomutase, barley dehydrin, and wheat germin genes. The expression of genes putatively associated with sugar metabolism and osmotic stress may be a requirement for freezing tolerance in plant cells.

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